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REVIEW

NADPH oxidases as a source of oxidative stress and molecular target in ischemia/reperfusion injury

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Abstract Ischemia/reperfusion injury (IRI) is crucial in the pathology of major cardiovascular diseases, such as stroke and myocardial infarction. Paradoxically, both the lack of oxygen during ischemia and the replenishment of oxygen during reperfusion can cause tissue injury. Clinical outcome is also determined by a third, post-reperfusion phase characterized by tissue remodeling and adaptation. Increased levels of reactive oxygen species (ROS) have been suggested to be key players in all three phases. As a second paradox, ROS seem to play a double-edged role in IRI, with both detrimental and beneficial effects. These Janus-faced effects of ROS may be linked to the different sources of ROS or to the different types of ROS that exist and may also depend on the phase of IRI. With respect to therapeutic implications, an untargeted application of antioxidants may not differentiate between detrimental and beneficial ROS, which might explain why this approach is clinically ineffective in lowering cardiovascular mortality. Under some conditions, antioxidants even appear to be harmful. In this review, we discuss recent breakthroughs regarding a more targeted and promising approach to therapeutically modulate ROS in IRI. We will focus on NADPH oxidases and their catalytic subunits, NOX, as they represent the only known enzyme family with the sole function to produce ROS. Similar to ROS, NADPH oxidases may play a dual role as different NOX isoforms may mediate detrimental or protective processes. Unraveling the precise sequence of events, i.e., determining which role the individual NOX isoforms play in the various phases of IRI, may provide the crucial molecular and mechanistic understanding to finally effectively target oxidative stress.

Keywords NADPH oxidases · NOX · Ischemia/reperfusion injury · Oxidative stress · ROS

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Introduction

Stroke and myocardial infarction (MI) are two major causes of death and disability in Western countries. Both are caused by organ ischemia followed by varying degrees of reperfusion. Reactive oxygen species (ROS) are thought to be the key players in this ischemia/reperfusion injury (IRI) [1, 2]. Consequently, treatment of IRI should be directed to these key players. The untargeted use of antioxidants, however, failed to show any clinical benefits after stroke [3, 4] or MI [5, 6]. Indeed, specifically targeting the pathological source of ROS may provide a better therapeutic approach. Among such potential ROS sources, NADPH oxidases are the only known enzymes with the sole function of producing ROS. In the cardiovascular system, NAPDH oxidases account for a major part of the ROS formed, not only during IRI but also under physiologic conditions [7]. Here, we review the pathophysiology of IRI, focusing on NADPH oxidases as new potential targets for therapeutic interventions.

Ischemia/reperfusion injury: two paradoxes

During ischemia or the subsequent reperfusion, damage can occur in cells or tissues, which often is irreversible. This damage is referred to as IRI [1, 8]. IRI can be divided into three phases.

During the first, ischemic phase, interruption of the blood flow to an organ causes a (temporary) lack of oxygen and nutrients [1, 2, 8, 9]. The generation of adenosine triphosphate (ATP) via oxidative phosphorylation is disturbed during ischemia, causing cells to alter their metabolism. The reduced availability of ATP limits the activity of the ATP-dependent Na^+/K^+ pump [1, 2], which constraints the outflow of calcium via the Na^+/Ca^{2+} exchanger [8]. This results in calcium overload, causing structural disorganization as well as apoptotic and necrotic death of cells [2, 8, 9]. In addition, chemokines and adhesion molecules promote a proinflammatory state [1, 2, 10]. Moreover, the altered cellular metabolism leads to an accumulation of precursors of oxidative phosphorylation, and it causes damage to or conformational changes of enzymes such as xanthine oxidase. These latter processes are not directly detrimental to cells, but they become important in the next phase of IRI by increasing ROS generation [1, 8, 9].

The second phase of IRI constitutes the reestablishment of blood flow and thus the reintroduction of oxygen. Although oxygen is needed for survival, it can also be detrimental because it can be converted to ROS such as hydrogen peroxide (H_2O_2) and superoxide (O_2^-) [1]. ROS can directly damage cells via a number of mechanisms, e.g., by influencing the opening probability of the mitochondrial permeability transition pore [11], by causing lipid peroxidation [1], by activating matrix metalloproteinases [12], or by oxidizing DNA [12]. However, ROS may also damage cells in a more indirect manner: They can, e.g., interact with nitric oxide (NO), fatty acids, or free iron (Fenton reaction). This often results in the formation of even more cytotoxic substances, such as peroxynitrite, peroxyl radicals, and hydroxyl radicals [11]. In addition, ROS can enhance the inflammatory response by the upregulation of chemokines and adhesion molecules [1, 10]. Thus, the reperfusion phase can be seen as a "double-edged sword" [13]: On one hand, there is a cellular demand for the replenishment of oxygen, while on the other hand, this oxygen results in ROS formation. The paradoxical role of oxygen in the first two phases is called the "oxygen paradox" [9].

The second ROS paradox shows up in a later and more chronic phase of IRI, the post-reperfusion phase. In contrast to their detrimental role in the acute phases, here, ROS affect several tightly regulated processes that lead to an optimal environment for survival: Angiogenesis is induced by ROS through the upregulation or activation of vascular endothelial growth factor (VEGF) and hypoxia-inducible factor (HIF) [14, 15]. The proliferation and differentiation of vascular smooth muscle cells (VSMC), needed for vascular remodeling, are also affected by ROS [14, 15]. These beneficial roles of ROS are also seen during permanent ischemia.

NADPH oxidases as a source of ROS in IRI

Most sources of ROS (see the "Other sources of ROS in IRI" section) only generate ROS "by accident," i.e., as a by-product of the metabolism they are involved in or during nonphysiologic conditions, such as ischemia [16–21]. NADPH oxidases stand out from these sources of ROS, as they constitute the only enzyme family with the sole function to produce ROS, not only in disease, but also in physiology [22]. NADPH oxidases are abundantly expressed in the vasculature, where they are emerging as the key producers of ROS [7, 23–26]. Depending on the phase of IRI, NADPH oxidases can be either detrimental or protective. Thus, NADPH oxidases also have a double-edged role, similar to their substrate, oxygen.

Interestingly, several drugs used to treat cardiovascular diseases regulate the expression and activity of NADPH oxidases. For example, statins prevent the translocation of the NADPH oxidase subunit Rac1 to the cell membrane [27]. This translocation is one of the essential steps in the activation of some NOX isoforms [28]. Thus, some of the pleiotropic effects of statins can be attributed to their ability to decrease NADPH oxidase-mediated ROS production [27, 29, 30]. Angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARB) reduce angiotensin II formation and angiotensin II-mediated AT1 receptor activation, respectively. Since angiotensin II signaling is a potent stimulus for NADPH oxidase expression and activity [29, 30], ACE inhibitors and ARB decrease NADPH oxidase expression and activation.

Biochemistry of NADPH oxidases

NADPH oxidases are multiprotein complexes. Seven isoforms of their catalytic subunits exist, annotated as NOX1–5 and DUOX1–2 (recently also termed as NOX6–7). They contain six or seven transmembrane spanning domains, respectively. They catalyze the electron transport from NADPH to molecular oxygen, thereby producing ROS [7, 22, 31, 32]. All isoforms have two heme groups, an FAD and a NADPH binding site [26]. NOX2 was the first described isoform. It was discovered in neutrophils, where it mediates oxidative burst and is thus important for innate host defense [22–24, 28]. In the vasculature, NOX2 is active in almost all vascular wall cells [33, 34]. In the cardiovascular system, next to NOX2, NOX1, NOX4, and NOX5 are also expressed. The latter, however, is not present in rats and

mice [24, 28, 32]. NOX1 is expressed in endothelial cells (EC), VSMC, and adventitial fibroblasts [35]. NOX4, the most abundantly expressed isoform, is found in EC and VSMC [24, 35]. Its expression and activity seem to be especially prominent in the cerebral vasculature [36], with gender-specific differences [37]. NOX5 is located in vascular EC [38] and VSMC [39]. The NOX isoforms not only vary in their expression pattern but also in their subcellular localizations. For example, NOX4, is found in the nucleus and might be present in mitochondria [40], making NOX4 a potential source of mitochondrial ROS. NOX1 colocalizes with caveolin-1 [41], associating it with caveolae and eNOS uncoupling (see below) [42]. Furthermore, the various NOX isoforms require different cytosolic subunits for activity, such as activator and organizer proteins or Rac1 [43, 44]. NOX4 stands out from the other NOX isoforms as it is constitutively active and produces H_2O_2 as its main product [28]. An important feature of NOX5 is that it does not need any of the cytosolic subunits. Importantly, it is directly activated by calcium through calcium binding to its EF hand motifs [24]. NOX6–7 (formerly called DUOX1–2) play probably no (major) role in the vasculature. Figure 1 and Table 1 give an overview of the characteristics of the isoforms expressed in the cardiovascular system.

Validation of NADPH oxidases as therapeutic targets for IRI

NADPH oxidases during acute ischemia

NADPH oxidases have been proposed to be oxygen sensors and thus to be involved in the ischemic phase of IRI (Fig. 2) [45–48]. They are also likely to interact with the HIF pathway (see below) [49–55].

Oxygen sensing is the endogenous ability of tissues to respond to changes in oxygen tension. It occurs in specialized cells such as the glomus cells of the carotid body and the neuroepithelial bodies in the lung. While NADPH oxidases are expressed in these tissues [47, 48], a true functional role in oxygen sensing is disputed [47, 48, 56–58]. In this review, we focus on true IRI; discussing the mechanisms of oxygen sensing in more detail is beyond the scope of this review.

First evidence for a role of NADPH oxidases in ischemia comes from studies showing an altered expression on mRNA or protein levels of NADPH oxidases caused by a

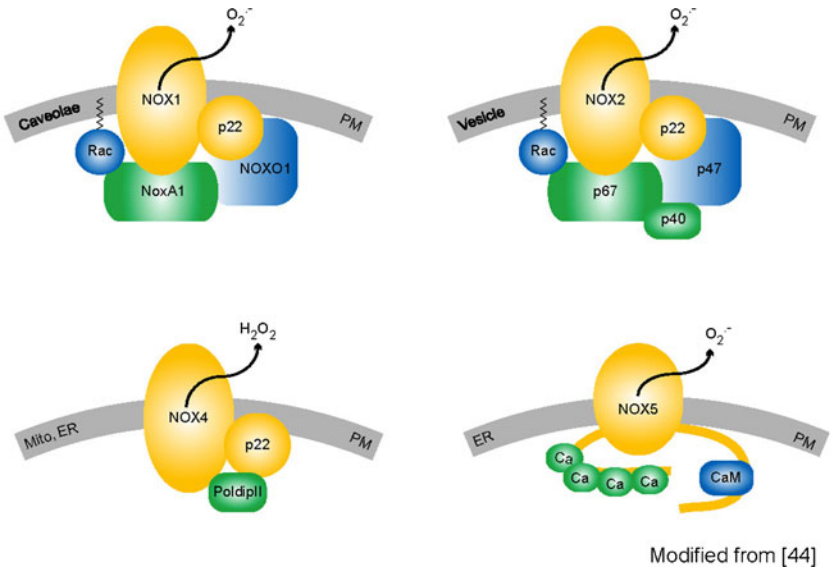

Fig. 1 The four vascular NOX isoforms need different subunits for their activation, have different subcellular localizations, and produce different types of ROS. NOX2 is the first described isoform of the NADPH oxidase family. It consists of a six-transmembrane-spanning catalytic domain. The membrane-bound subunit p22phox and cytosolic subunits p47phox, p67phox, and p40phox need to assemble for NOX2 activation. In addition, the small G protein Rac binds to this complex. NOX2 is mainly localized in intracellular vesicles, but also in plasma membranes (*PM*). The basic structure of NOX1 is similar to NOX2. However, NOX1 interacts with the p47phox and p67phox analogs, NOXO1 and NOXA1, although at least in recombinant systems, it is also active upon interaction with p47phox and p67phox (not shown). NOX1 activity also depends on p22phox and Rac. Similar to NOX2, NOX1 is located in the PM. In addition, it was suggested to be located in caveolae. NOX3 appears not to be expressed in the vasculature but in the inner ear [28, 31]. NOX4 is different from the other vascular isoforms in that it only interacts with p22phox but does not rely on other cytosolic subunits for its activation. It was suggested that NOX4 is not only located in the plasma membrane, but also in membranes of the endoplasmic reticulum (*ER*) and in mitochondria. NOX4 is constitutively active, but can be further activated by several factors such as angiotensin or growth factors. PolDip2 has been described as a binding protein that can activate NOX4 [152]. NOX4 has been suggested to also or even predominantly generate hydrogen peroxide (H_2O_2). NOX5 has mainly been found in the ER in addition to its localization in the PM. It produces superoxide and is active without any subunits. An increase in calcium activates NOX5 mediated by its calcium binding domains, i.e., the EF hands. In addition, the C terminus of NOX5 has a calmodulin-binding domain

Table 1 Specifications of NADPH oxidase isoforms (adapted from [88])

Isoform	Regulators	Stimulators	Tissue distribution	Cellular distribution	Subcellular localization
NOX1	$p22^{phox}$, NOXO1, NOXA1, Rac, PDI, Hsp90, hypoxia	PDGF, prostaglandin 2α, AT-II	Brain, vessels, colon, stomach, uterus, placenta, prostate, retina	Neurons, astrocytes, microglia VSMCs, epithelial cells, osteoclasts	ER, sarcoplasmic caveolae, endosomes
NOX2	$p22^{phox}$, $p67^{phox}$, $p47^{phox}$, Rac, Hsp90, hypoxia	Phagocyte stimulation, IFN-γ, hypoxia, AT-II	Brain, vessels, liver, muscle	Neutrophils	Intracellular compartments (granules)
				Monocytes, macrophages, T cell, microglia, astrocytes, ECs, fibroblasts, cardiac myocytes, hepatocytes, hematopoietic stem cells, neurons, smooth muscle cells	Plasma membranes, membranes of synaptic sites, perinuclear cytoskeleton
NOX4	p22phox, PolDip2, PDI, hypoxia	ER stress, shear stress, TGF-β1, TNF-α	Ubiquitous, especially kidney, vessels, lung, bone	Neurons, astrocytes, ECs, VSMCs, fibroblasts, mesangial cells, keratinocytes, osteoclasts, hepatocytes	Focal adhesions, ER, nucleus, mitochondria
NOX5	No subunits, but calcium-sensitive, Hsp90	Calcium	Testis, spleen, kidney, lymphatic tissue, uterus, vessels	ECs, VSMCs, lymphocytes	Unknown

AT-II angiotensin II, *ECs* endothelial cells, *ER* endoplasmic reticulum, H_2O_2 hydrogen peroxide, *Hsp90* heat shock protein 90, O_2^- superoxide, *IFN-γ* interferon-γ, *PDI* protein disulfide isomerase, *PolDip2* polymerase (DNA-directed) delta-interacting protein, *TGF-β1* transforming growth factor-β1, *TNF-α* tumor necrosis factor alpha, *VSMCs* vascular smooth muscle cells

change in oxygen tension. In pulmonary artery smooth muscle cells (PASMC), EC, pulmonary epithelial cells, and PC12 cells, a cell line from pheochromocytoma of rat adrenal medulla [51, 53, 54], either NOX1, NOX2, or NOX4 mRNA and protein levels are upregulated during hypoxia. In vivo, an upregulation of NOX4 was also shown in mouse lungs after hypoxia [51, 59]. Similarly, mRNA levels of the NADPH oxidase subunits $p22^{phox}$, $p47^{phox}$, and $p67^{phox}$ increased in response to hypoxia in human umbilical vein endothelial cells (HUVEC) [60]. In the brain of rats and mice, upregulation of NOX2, NOX4, and p47phox mRNA levels was reported after stroke induced by ischemia/reperfusion (I/R) [61–63]. An increase in NOX2, NOX4, $p22^{phox}$, and $p67^{phox}$ mRNA was also found after MI in mice [64–66]. Furthermore, NOX4 mRNA and protein levels were upregulated in peripheral muscles after hindlimb ischemia in mice [67]. Taken together, the aforementioned studies indicate that NOX isoforms are upregulated at the mRNA level. However, a concomitant increase at the protein or functional level has not always been reported. Therefore, further studies are necessary.

The effects of hypoxia on NADPH oxidases and reciprocal effects of NADPH oxidases on hypoxic signaling pathways seem to be mediated by different regulatory pathways, some of them involving hypoxia-inducible transcription factors of the HIF family [68]. HIFs are composed of an oxygen-sensitive α-subunit and a constitutively expressed β-subunit also termed aryl hydrocarbon receptor nuclear translocator [69].

Interactions between NADPH oxidases and HIF proteins have been shown for different NOX isoforms and NADPH oxidase subunits [68, 70]. ROS derived from NADPH oxidases can upregulate HIF-1α mRNA and protein levels [71]. In VSMC, HIF-1α is induced by thrombotic factors. This induction and its further activation depend on Rac1 and $p22^{phox}$ [52, 72]. Further data indicate that NOX2 and NOX4 play a role in regulating HIF-1α levels in EC, thereby modulating angiogenic activity [73]. In PASMC, NOX4 induced HIF-1α and HIF-2α levels by interfering with the activity of the HIF-α-modifying prolyl hydroxylases [50]. These data indicate that NADPH oxidases can modulate HIF activity by several mechanisms, including transcriptional upregulation of HIF-1α through the activation of NFκB [71]. In addition, NADPH oxidases have been reported to mediate posttranslational stabilization of HIF-1α proteins by interfering with the HIF-degrading machinery [50].

Both transient and stable overexpression of NOX1 in A549 cells resulted in increased HIF-1-dependent target gene expression [53]. Also, increased HIF-1α protein was found both during hypoxia and normoxia in these NOX1-transfected cells [53].

On the other hand, expression of active Rac1 decreased nuclear HIF-1α levels in HepG2 cells and primary rat hepatocytes under hypoxia [74], while opposite effects were reported in Hep3B cells [75]. The different NOX isoforms/

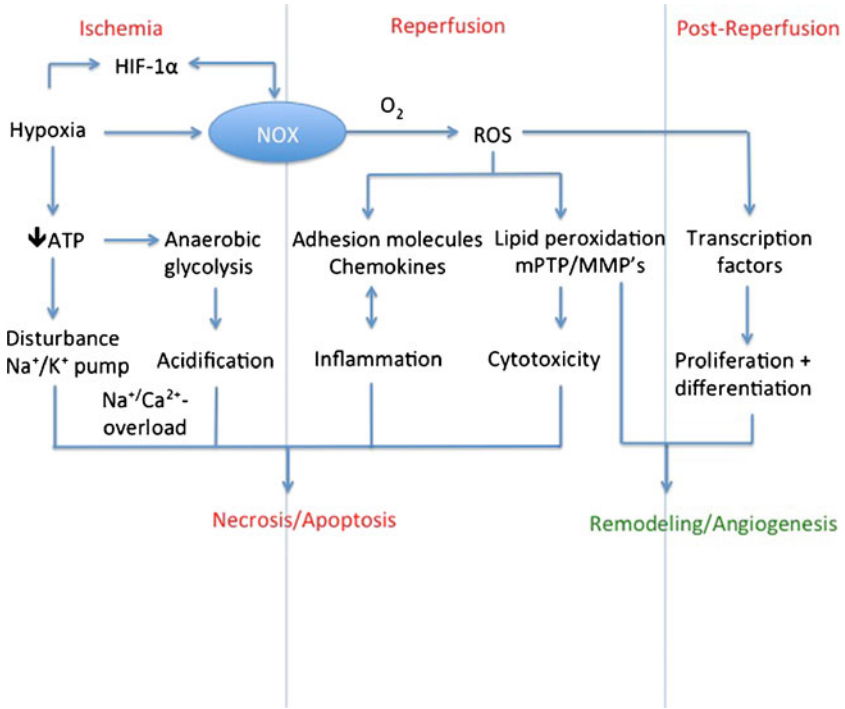

Fig. 2 The role of NADPH oxidases in the three phases of IRI. During the ischemic phase, the lack of oxygen results in decreased ATP production, increasing the calcium concentration, which ultimately results in necrosis. In addition, the drop in oxygen upregulates NADPH oxidases via stimulation of HIF-1α. NADPH oxidases can also activate HIF-1α, initiating a positive feedback loop. When a reintroduction of oxygen occurs during the reperfusion phase, NADPH oxidases produce large amounts of ROS. These ROS then enhance the inflammatory response by upregulation of adhesion molecules and chemokines, which activate and attract leucocytes. Inflammation and edema ensue. ROS can also have direct cytotoxic effects. Together with the inflammation, this ultimately results in necrosis and apoptosis of cells. ROS production also occurs during the post-reperfusion phase. In this phase, a certain level of ROS is needed to ensure a proper stimulation of angiogenesis via VEGF activating EC and remodeling via the NFκB pathway activating VSMC. This leads to a better environment for tissue survival

subunits might thus have specific roles, and/or the redox sensitivity of different cells and tissues might be specific.

NADPH oxidases not only regulate HIF-1α and HIF-2α, but HIF can vice versa also affect NADPH oxidases. In mice with a heterozygous defect in HIF-1α and thus reduced HIF-1α levels, NOX2 mRNA and protein did not increase in the cortex and brainstem after exposure to intermittent hypoxia, while in wild-type (WT) mice, NOX2 was upregulated [54]. Fibroblasts from HIF-1α heterozygous mice had a lower basal HIF-dependent reporter gene activity, and NOX2 mRNA levels did not increase after hypoxia, but they did increase in fibroblasts from WT mice [54]. Similarly, in endothelial-specific HIF-1α knockout (KO) mice, NOX2 mRNA expression and protein levels were reduced even when adding the NOX2-stimulator urotensin-II [73].

Overexpression of HIF-1α in PC12 cells also increased NOX2 mRNA and protein levels as well as NADPH oxidase activity after intermittent hypoxia [55]. Importantly, a functional HIF binding site in the proximal promoter of the NOX2 gene was recently identified [73], and thus, NOX2 was added to the list of genuine HIF-1 target genes. As such, HIF-1 can regulate the expression of NOX2 and thereby ROS production by NOX2. For NOX4, a decrease of its mRNA levels in PASMC during hypoxia after treatment of the cells with shRNA against HIF-1α was reported [51]. Vice versa, overexpression of HIF-1α resulted in elevated NOX4 mRNA and protein levels. ROS production also increased after overexpression of HIF-1α, but only when NOX4 was present [51]. Similarly to NOX2, a functional HIF binding site was identified in the NOX4 promoter, thus adding also NOX4 to the list of direct HIF target genes [51].

These findings suggest that ROS-dependent activation of HIF can induce the expression of several NADPH oxidase subunits under normoxic conditions, thus promoting ROS-mediated signaling pathways. Under low oxygen conditions, such as ischemia, the rapid stabilization and activation of HIF may be the primary event responsible for the upregulation of NADPH oxidases. This process may help cells and tissues to adapt at least partially to the decreased substrate availability under these conditions, thereby also allowing a certain restoration of ROS levels under hypoxia. However, whether NADPH oxidases can also be activated under hypoxia without concomitant increase in subunit availability is not clear and precise mechanisms and interactions of NADPH oxidase-derived ROS under these conditions still need to be elucidated.

NADPH oxidases during reperfusion

Upon reintroduction of oxygen, NADPH oxidases are believed to generate large amounts of ROS, which have direct cytotoxic effects [11, 12] (Fig. 2). For example, after I/R of the lung or the brain, lipid peroxidation, protein nitration, and oxidative DNA damage were smaller in NOX2 KO [76] or $p47^{phox}$ KO mice compared to WT mice [77, 78]. Similar results were found when apocynin, an unspecific NADPH oxidase inhibitor [30, 79], was applied to WT mice [76, 78]. Apocynin and diphenylene iodonium (DPI), another unspecific NADPH oxidase inhibitor, also reduced the increases in lipid peroxidation, cell death, and apoptosis after I/R in H9c2 cells [80, 81]. However, these results should be interpreted with caution because of the lack of specificity of these inhibitors: apocynin has antioxidant properties [79] and DPI is a general flavoprotein inhibitor [82]. The more specific NADPH oxidase inhibitor VAS2870 given to WT mice 2 and 12 h after cerebral I/R, which was induced by transient middle cerebral artery occlusion, decreased infarct size, ROS levels, tissue nitration, apoptosis, and brain edema. VAS2870 also improved the functional outcome in WT mice after the induction of ischemic strokes [62]. NOX4 KO mice showed the same decreases in damage as VAS2870-treated WT mice, while treatment of NOX4 KO mice with VAS2870 did not have an additional protective effect [62]. This strongly suggests that NOX4 contributes to cerebral IRI. Together, these studies show that NADPH oxidases-derived ROS, particularly NOX4, are likely to be involved in the direct cytotoxic effects of IRI in the brain.

Evidence for the role of other NOX isoforms in cerebral IRI is contradictory. No effect of NOX1 or NOX2 in brain IRI was found in the study mentioned above [62]. Two other studies showed no role [83] or a detrimental role [84], respectively, of NOX1 in stroke. Data concerning the role of NOX2 are also conflicting [62, 76, 77, 84–87]. For a more detailed discussion of the role of NADPH oxidases in brain I/R, we refer to [88].

NADPH oxidases can also indirectly cause damage by enhancing the inflammatory response [22]. Neutrophils, which express NOX2, are the primary source of ROS after heart I/R in dogs [89]. Further, neutrophil infiltration was reduced after brain I/R in NOX2 KO mice compared to WT mice. Similarly, neutrophil infiltration was attenuated in the lungs of $p47^{phox}$ KO mice or of mice treated with apocynin [76, 78]. However, again, apocynin is not specific for NADPH oxidases. Furthermore, after lung I/R, proinflammatory cytokine levels including TNF-α, IL-6, IL-1β, and MCP-1 in lungs were lower in $p47^{phox}$ KO mice compared to WT mice [78]. This was also observed in the brain after I/R [76]. Here, the ischemic stroke induced the disruption of the blood–brain barrier (BBB), which contributes to inflammation via increased permeability and subsequent tissue swelling after brain I/R. This BBB disruption was reduced in NOX2 KO mice compared to WT mice [86].

In an isolated perfused lung I/R model, edema and vascular permeability were reduced in lungs from NOX2 KO mice, but neither in lungs from NOX1 KO mice nor in NOX4 KO mice [90]. Together, these data suggest a role of NOX2 or $p47^{phox}$ in the inflammatory reaction and chemotaxis during IRI. The regulation of different cytokines in different organs suggests a cell-specific or organ-specific effect of NOX2. With respect to the other NOX isoforms, no solid data on their involvement in inflammation and chemotaxis after IRI are available.

NADPH oxidases during post-reperfusion and chronic ischemia

ROS are not only toxic during acute IRI, they are also key signaling molecules in pathways mediating cell proliferation and differentiation [7, 22, 91–93], which are needed for both angiogenesis and remodeling (Fig. 2). During the post-reperfusion period or during chronic ischemia, NADPH oxidases might thus have a protective role, showing the second edge of the NADPH oxidase sword [94–97]. On one hand, ROS can mediate autophosphorylation of vascular endothelial growth factor receptor 2 (VEGFR2), which leads to the activation of downstream signaling pathways [96]. In addition, by interacting with HIF-1α, ROS can activate VEGF and other targets, which protect and activate vascular proliferative responses such as plasminogen activator inhibitor-1 (PAI-1) [68, 70, 71].

On the other hand, VEGF can activate NADPH oxidases via Rac1, leading to increased ROS production [98]. This feedback loop ultimately results in increased VEGF-induced angiogenesis. In nonvascular cells, there is also a negative feedback loop involved, in which Rac1 inhibits HIF-1 and PAI-1 and thus decreases angiogenesis [74].

The nonspecific NADPH oxidase inhibitor, DPI, and the antioxidant, *N*-acetylcysteine, decreased VEGF-induced ROS levels in HUVEC, as did NOX2 antisense constructs [98]. $p47^{phox}$ downregulation in HUVEC also led to decreased VEGF-mediated phosphorylation of downstream mediators [99]. VEGF-induced neovascularization in a sponge implant model was also reduced in NOX2 KO mice compared to WT mice [98]. Together, these data clearly point to a role of NADPH oxidases in the VEGF signaling pathway in vascular cells at several levels. However, the importance of NOX2 for angiogenic responses is not limited to VEGF signaling. Recently, it was reported that the vasoactive peptide urotensin-II can also induce angiogenesis, and this response was inhibited in vitro by downregulating NOX2 in EC using shRNA. Similarly, vascular sprouting into a Matrigel plug was disrupted in NOX2 KO mice, but not in WT mice [73].

After hindlimb ischemia, flow recovery and capillary density were reduced in NOX2 KO mice [100, 101]. However, there is also evidence for a normal collateral growth in NOX2 KO mice, with only a dysfunction of the newly formed vessels, resulting in compromised perfusion [102]. In addition, when adding oxidative stress triggers, such as smoking or diabetes, the impairment of new vascularization was less pronounced in NOX2 KO mice compared to WT mice [103, 104]. Thus, while NOX2 clearly seems to be involved in promoting angiogenesis in vivo, it is the specific environment (i.e., oxidative stress or not) that finally determines the overall impact of NOX2 on these responses.

During remodeling in the chronic phase of IRI, NOX2 seems to play a detrimental role. NOX2 or $p47^{phox}$ KO mice subjected to MI displayed less dilatation of the left ventricle, less hypertrophy, and less interstitial fibrosis along with an increased survival after 4 weeks [64, 65]. Interestingly, infarct sizes did not differ between KO and WT mice both after 24 h and 4 weeks [64, 65]. Others did not find any difference between NOX2 KO and WT mice in infarct size, apoptosis, collagen content, or inflammation after MI [66, 105]. However, some studies neither used littermates as control mice nor sham-operated animals. This is relevant when interpreting these results. Furthermore, differences in mouse strains can influence heart remodeling [106]. Also, $p47^{phox}$ KO and $p47^{phox}$ heterozygous mice had similar infarct sizes and left ventricular functions 24 h after I/R of the heart [107]. However, 24 h of reperfusion in the heart can be rather regarded as an acute reperfusion phase; thus, these results suggest that there is no role for NOX2 and $p47^{phox}$ in acute heart IRI. Rather, there may be an effect in the post-reperfusion phase. Although the data are contradictory, most studies suggest a rather detrimental role of NOX2 in long-term remodeling after MI.

NOX1 has been shown to be involved in angiogenesis in tumors [108], but data in I/R models are missing. NOX1-overexpressing transgenic mice showed an increased hypertrophy of VSMC in response to angiotensin II [109]. Moreover, NOX1 may be involved in cell growth and transformation, not only in the vasculature but also in tumor-forming cells [110, 111].

For NOX4, data suggest a protective role in the chronic phase of IRI. siRNA against NOX4 reduced tube formation and wound healing responses in HMEC and HUVEC and also inhibited both basal and VEGF-induced cell migration or proliferation [67, 112]. NOX4 overexpression increased these responses [67, 112]. In line, similar responses of cell migration and proliferation were found after overexpressing or silencing NOX4 in human and bovine EC grown on Matrigel [67]. However, studies using siRNA against NOX4 should be interpreted with caution, since not all siRNAs specifically downregulate NOX4 and some may also affect other NOX isoforms [113].

In mice injected with adenoviral NOX4 into the hindlimb, blood flow was increased after ligation of the femoral artery compared to nontreated animals [67]. Accordingly, blood flow restoration in NOX4 KO mice was reduced after hindlimb ischemia [114]. Furthermore, aortas of endothelial-specific NOX4 transgenic mice displayed increased sprouting ex vivo [67]. Thus, a protective role for NOX4 in angiogenesis during chronic ischemia is likely.

In addition, an involvement of NOX4 in the regulation of VSMC growth and differentiation was suggested [91, 93]. In wound healing and embryogenesis, maintenance of VSMC differentiation might be a beneficial effect of NOX4 [91]. However, in IRI and cardiac pressure overload, NOX4 in cardiomyocytes and cardiac fibroblasts may have a more ambivalent role: They seem to stimulate proliferation and differentiation of cardiac fibroblasts into myofibroblasts, which may lead to fibrosis, cardiac remodeling, and heart failure [92, 115]. In human PASMC, NOX4 also mediated proliferation in response to hypoxia, but also to factors such as transforming growth factor β1 or urotensin-II, which can contribute to vascular remodeling in different disease states [51, 116, 117]. Moreover, hypertrophic stimuli such as angiotensin II and pressure overload increased NOX4 expression in cardiac myocytes [118]. Aged mice overexpressing NOX4 in the heart showed increased fibrosis and apoptosis of cardiomyocytes [118]. Interestingly, overexpression of HIF-1α increased proliferation in PASMC, but not when NOX4 was depleted by shRNA [51]. Thus, an additional role of HIF-1α in the VSMC proliferative response seems likely. Altogether NOX4 seems to be involved in regulating the growth and differentiation of VSMC and cardiac cells, which in the post-reperfusion phase might lead to remodeling and formation of fibrosis. Unfortunately, there are no in vivo data on the effect of NOX4 on the long-term remodeling after IRI. The in vivo and in vitro studies discussed are summarized in Tables 2 and 3, respectively.

Other sources of ROS in IRI

As already mentioned, other sources of ROS distinct from NADPH oxidases generate ROS under nonphysiological conditions. This indicates that these sources might also play a role in IRI. For example, xanthine oxidoreductase (XOR) catalyses the formation of xanthine and uric acid from hypoxanthine. It can exist in two different forms, xanthine dehydrogenase that transfers electrons to NAD^+ and xanthine oxidase, which cannot bind NAD^+, thus transferring electrons directly to molecular oxygen [119]. Under conditions of inflammation or oxidative stress, xanthine dehydrogenase can be posttranslationally modified to the ROS-producing xanthine oxidase [20, 119]. The evidence for a

Table 2 In vivo studies on the role of NADPH oxidases in IRI

Heart								
Animal model	Ischemic model	Time point investigated	Outcomes Cardiac dilatation	Cardiac dysfunction	Infarct size	Survival	Inflammation	Reference
$p47^{phox}$ KO vs WT mice	LAD ligation	4 week	↓	↓	Ø	↑	n.a.	[64]
$p47^{phox}$ KO vs $p47^{phox}$ Het mice	Transient LAD ligation (30 min)	24 h	Ø	Ø	Ø	n.a.	↑	[107]
NOX2 KO vs WT mice	LAD ligation	8 week	Ø	Ø	Ø	↓	n.a.	[105]
NOX2 KO vs WT mice	LAD ligation	4 week	↓	↓	Ø	Ø	n.a.	[65]
NOX2 KO vs WT mice	LAD ligation	4 week	n.a.	n.a.	Ø	n.a.	Ø	[66]
Brain								
Animal model	Ischemic model	Time point investigated	Outcomes Infarct size	Neurological outcome	Inflammation	BBB dysfunction		Reference
NOX1 KO vs WT mice	tMCAO (30 min)	24 h	Ø	Ø	n.a.	n.a.		[83]
NOX1 KO vs WT mice	tMCAO (60 min)	24 h	Ø	Ø	n.a.	n.a.		[62]
NOX1 KO vs WT mice	tMCAO (60 min)		↓	↑	n.a.	↓		[84]
NOX2 KO vs WT mice	tMCAO (60 min)	24 and 72 h	↓	n.a.	↓	n.a.		[77]
NOX2 KO vs WT mice	tMCAO (75 min)	24 and 72 h	↓	↑	↓	n.a.		[76]
NOX2 KO vs WT mice	tMCAO (120 min)	1/2/24 h	24 h, ↓; 2 h, Ø	n.a.	n.a.	1 h, ↓		[86]
NOX2 KO vs WT mice	tMCAO (60 min)	24 h	Ø	Ø	n.a.	n.a.		[62]
NOX2 KO vs WT mice	tMCAO (25 min)	72 h	↓	n.a.	n.a.	n.a.		[150]
NOX2 KO vs WT mice	tMCAO (120 min)	24 h	↓	n.a.	Ø	n.a.		[87]
NOX4 KO vs WT mice	tMCAO (60 min)	24 h	↓	↑	n.a.	↓		[62]
	pMCAO	24 h	↓	↑	n.a.	n.a.		
	PT	24 h	↓	n.a.	n.a.	n.a.		
WT mice treated with VAS2870	TMCAO	24 h	↓	↑	n.a.	n.a.		
Hindlimb								
Animal model	Ischemic model	Time endpoint	Outcomes Blood flow recovery	Capillary density		Ischemic muscle injury		Reference
NOX1 KO vs WT mice	FA ligation	2 weeks	↑	n.a.		n.a.		[114]
NOX2 KO vs WT mice	FA excision	2 weeks	↓	n.a.		↑		[102]
NOX2 KO vs WT mice	FA ligation	3 weeks	↓	↓		n.a.		[104]
Diabetic NOX2 KO vs diabetic WT mice			↑	↑		n.a.		
NOX2 KO vs WT mice	FA excision	2 weeks	↑	↑		n.a.		[103]
NOX2 KO vs WT mice after chronic smoke exposure			↑	↑		n.a.		
NOX2 KO vs WT mice	FA ligation	2 weeks	↓	n.a.		n.a.		[114]
NOX2 KO vs WT mice	FA excision	1 week	↓	↓		n.a.		[101]
NOX2 KO vs WT mice	FAV excision	2 weeks	↓	n.a.		n.a.		[100]
WT mice after adenoviral NOX4 injection vs WT mice	FA ligation	4 weeks	↑	↑		n.a.		[67]
NOX4 KO vs WT mice	FA ligation	2 weeks	↑	n.a.		↑		[114]
Inducible NOX4 KO vs WT mice			↑	n.a.		↑		
Lung								
Animal model	Ischemic model	Time point investigated	Outcomes Edema	Kfc		Pulmonary function		Reference
NOX1 KO vs WT mice	Isolated perfused lung ischemia (90 min)	30/60/90 min	Ø	Ø		n.a.		[90]
NOX2 KO vs WT mice			↓	↓		n.a.		
NOX4 KO vs WT mice			Ø	Ø		n.a.		
$p47^{phox}$ KO vs WT mice	Lung hilar ligation (60 min)	120 min	↓	n.a		↑		[78]

n.a. not applicable, *BBB* blood–brain barrier, *FA* femoral artery, *LAD* left anterior descending coronary artery, *pMCAO* permanent middle cerebral artery occlusion, *tMCAO* transient middle cerebral artery occlusion, *Ø* no change

Table 3 In vitro studies on the role of NAPDH oxidases in IRI

Cell/tissue	Treatment	Outcome	Reference
ROS production and NOX expression			
H9c2 cardiac cells	I/R	↑ ROS, ↑ $p47^{phox}$ protein, ↑ NOX2 protein	[80]
	I/R+DPI or apocynin	↓ ROS increase	
H9c2 cardiac cells	Metabolic inhibition (ischemia)	↑ ROS, ↑ NOX2 mRNA, ↑ apoptosis	[81]
	Metabolic inhibition+DPI or apocynin	↓ ROS increase, ↓ apoptosis	
Human PASMC	Hypoxia	↑ NOX4 mRNA and protein and HIF-1α mRNA and protein	[51]
HepG2 cells	Hypoxia and overexpression HIF-1α	↑ Cell proliferation and ROS production	
HEK293	Hypoxia, overexpression HIF-1α and depletion NOX4 with shRNA	↓ of increased proliferation and ROS production	
Angiogenesis			
NIH 3T3 cells	Transfection of cells with NOX1,	↑ VEGF expression, ↑ H_2O_2 production	[108]
DU-145	then injection into athymic mice	↑ Growth of tumours with more vascularization	
NIH-3T3 cells	Transfection of cells with NOX1, then overexpression catalase	↑ Cell proliferation, ↑ H_2O_2 production, Reversion of NOX1 induced phenotype	[110]
Human and bovine EC	Adenoviral overexpression or knockdown NOX4	↑ Tube formation in overexpressing, ↓ tube formation in knockdown cells	[67]
Aortas from EC-specific NOX4 Tg and WT mice	–	↑ Capillary sprouting in Tg aortas	
HMEC and HUVEC	siRNA to NOX4	↓ Tube formation and ↓wound healing	[112]
HMEC	Adenoviral transfection NOX4	↓ EC migration and proliferation	
		↓ Tube formation but ↑wound healing	
HUVEC	NOX2 antisense	↓ VEGF induced ROS production, proliferation, and migration	[98]
HCAEC/HUVEC	$p47^{phox}$ siRNA	↓ VEGF mediated phosphorylation of VEGFR2	[99]
Human endothelial cells	Urotensin	↑ Formation capillary like structures in Matrigel	[73]
Mouse vena cava explants	Urotensin in NOX2 depleted cells	↓ Formation capillary like structures in Matrigel	
	Urotensin	↑ Sprouting in Matrigel	
Remodeling			
VSMC from aortas of SD rats	–	High NOX4 mRNA in differentiating cells, low NOX4 in dedifferentiating cells	[91]
	NOX4 siRNA/antisense	Low NOX1 mRNA in differentiating cells, high NOX1 in dedifferentiating cells	
		↓ Differentiation markers and ↓ contractile type stress fibers	
Human primary cardiac fibroblasts	TGF-β1	↑ NOX4 mRNA, Ø NOX1, NOX2 and NOX5 mRNA	[92]
	NOX4 siRNA	↓ Basal and TGF-β1 stimulated ROS production, ↓ SM α-actin mRNA and protein	
	NOX5 siRNA	Ø Basal and TGF-β1 stimulated ROS production	
Aortic VSMC from NOX1 Tg and WT mice	AT-II	↑ ROS production in Tg, blood pressure elevation in vivo higher in Tg, greater hypertrophy (aortic medial thickness) in Tg	[109]
Mouse ESC	siRNA against NOX4, shRNA	↓ Spontaneously beating EB's	[151]
Neonatal mouse cardiomyocytes	against NOX4 siRNA	Impaired cardiac myofibrillogenesis	
Neonatal rat cardiomyocytes	AT-II, TAC, and aging	↑ NOX4 protein expression	[118]
Cardiomyocytes from mice	NOX4 overexpression	Cell size Ø, ↑ apoptosis in overexpressing cells	
Human PASMC	Urotensin-II	↑ $p22^{phox}$ and NOX4 protein expression, ↑ROS production, ↓ of increased ROS production	[116]
	Urotensin-II and antisense vectors against p22phox and NOX4		
Human PASMC	TGF-β1	↑ NOX4 mRNA and protein expression and ROS production	[117]
	TGF-β1 and siRNA against NOX4	↓ TGF-β1 induced proliferation	

AT-II angiotensin II, *DU145* epithelial cell line derived from human prostate tumor, *EB* embryoid bodies, *ESC* embryonic stem cells, *HEK293* human embryonic kidney cells, *HCAEC* human coronary artery endothelial cells, *HepG2* human hepatoblastoma cells, *HMEC* human microvascular endothelial cells, *HUVEC* human umbilical vein endothelial cells, *NIH 3T3 cells* mouse embryonic fibroblasts, *PASMC* pulmonary artery smooth muscle cells, *SD rats* Sprague–Dawley rats, *siRNA* short interference RNA, *shRNA* short hairpin RNA, *riRNA* ribosomal RNA, *TAC* transient aortic constriction, *VEGF* vascular endothelial growth factor, *VSMC* vascular smooth muscle cells, *Ø* no change

role of XOR in IRI is controversial, showing both positive and negative roles as reviewed in [16, 20, 119].

NO synthase catalyses the formation of NO from L-arginine, using tetrahydrobiopterin (BH_4) as an essential, redox-sensitive cofactor. NOS, too, can have double-edged roles within the vasculature: NO is beneficial by mediating, for example, vasorelaxation, anticoagulant, and antiproliferative properties [10]. However, during IRI, ROS uncouple NOS by oxidizing BH_4 [120]. NOS itself then produces ROS instead of NO [10, 12, 121]. In addition, NO can react with superoxide to form toxic peroxynitrite [122, 123], causing further cell and tissue damage.

Mitochondria have also been described as being major contributors to ROS production in IRI, especially in the heart [17, 124–127]. Mitochondria form ROS via electron leakage from the electron transport chain during oxidative phosphorylation at different complexes [128] or via less common ways involving monoamine oxidases or p66Shc protein [18]. Similar to the case of NOS, ROS produced by mitochondria or by other sources can lead to a further increased production of ROS: ROS induced ROS release [129, 130]. Mitochondrial ROS have been shown to also play a role in ischemic preconditioning [131, 132].

Interplay between different sources of ROS can also be one of the mechanisms contributing to IRI [11, 22, 128]. NOX4, for example, has been shown to localize in mitochondria, indicating that NOX4 might produce mitochondrial ROS [40]. The ROS produced by NADPH oxidases can then trigger mitochondrial dysfunction, leading to increased ROS production and, vice versa, ROS produced by mitochondria can activate NADPH oxidases. This has recently been reviewed in more detail elsewhere [128].

Nevertheless, it has yet to be established which source(s) of ROS are the pathologically relevant ones in IRI. However, again, NADPH oxidases are the only enzymes with the sole function of generating ROS. Therefore, targeting NADPH oxidases seems a new promising therapeutic strategy, which seems superior compared to using antioxidants, as will be discussed in the following section.

Therapeutic strategies

Antioxidants

As ROS and oxidative stress are likely major players in IRI, attempts to prevent and treat related disorders with antioxidant supplements seemed plausible. Indeed, many clinical trials testing the effects of antioxidant application have been performed.

Some rather small clinical trials tested the acute or subacute therapeutic effects of antioxidants in patients with I/R-related disorders, with conflicting results. For example, in patients undergoing aortic cross-clamping, oral supplementation of vitamin E (60 mg for 8 days presurgery) prevented the overproduction of ROS and diminished muscle damage in the lower limbs [133]. However, such a preventive intervention is, by definition, excluded in post-infarct or post-stroke therapies. Nevertheless, in patients with diabetes, but not in nondiabetic patients, a reduction in 30-day mortality after acute MI was found when patients were treated with high doses of vitamin C (1,000 mg intravenously [IV] in 12 h, 400 mg orally) and vitamin E (200 mg, two times daily) for 30 days [6]. In contrast, human superoxide dismutase (IV bolus of 10 mg/kg followed by infusion of 0.2 mg/kg/min for 60 min) did not show any effect after acute MI in patients undergoing percutaneous transluminal coronary angioplasty [134]. In smokers with MI, oral supplementation of vitamin E (50 mg daily) did not reduce the incidence of recurrent MI [135]. Alarmingly, the administration of vitamin C (2 g IV 2 h prior to surgery) in patients undergoing elective I/R (aortic clamping) promoted iron-induced oxidative lipid damage via a Fenton-type reaction. The subsequent release of lactate dehydrogenase into the systemic circulation may have catalyzed the formation of second-generation radicals implicated in the regulation of vascular permeability and angiogenesis [136]. The reasons for the conflicting results are not known, but may be caused by the use of different antioxidants, doses, and timing as well as patient collectives. Nevertheless, as these studies only included relatively small patient numbers, they rather have a pilot character.

Many large and long trials tested antioxidant supplements for primary and secondary prevention of I/R events. Despite the huge patient numbers, study results are conflicting [3, 5, 6, 135, 137, 138]. However, several meta-analyses suggest that the application of antioxidants does not improve cardiovascular mortality and morbidity. In contrast, untargeted supplementation of antioxidants may even be harmful [5, 27, 29, 30, 139]. Thus, although ROS play an important role in IRI, antioxidants failed to prevent related disorders. Several explanations for this failure have been proposed. For example, antioxidants given orally might not penetrate into the vascular wall; scavenging of radicals can convert antioxidants to radicals themselves exerting pro-oxidant effects; and the timing of the application might not be right, since ROS already play a role in the initiation of cardiovascular diseases [27, 139]. Furthermore, antioxidants—if they reach the target tissue—cannot distinguish between good and bad ROS.

Thus, unraveling the role of ROS and their sources at different time points in IRI is crucial for designing the optimal therapeutic approach. Preventing the formation of disease-triggering ROS at the right time, instead of scavenging them after they have been formed, seems a better therapeutic approach.

NADPH oxidases as therapeutic targets in IRI

NADPH oxidases are crucial players in all phases of IRI. Thus, interfering with the NADPH oxidase function is a promising strategy for treating IRI. However, important issues need to be considered: First, timing is likely to be crucial, since NADPH oxidases and ROS play different roles, depending on the time course of IRI. Second, isoform-specific differences or organ/cell-specific effects have to be taken into account. Third, the amount of ROS is important, since low amounts of ROS are needed for physiologic functions such as regulation of cell growth and differentiation [11, 12], whereas increased levels surpass endogenous antioxidant defense and cause cell damage.

The underlying mechanisms of all these factors are only incompletely understood. During ischemia, NADPH oxidases interact with HIF [49–51, 53–55] and may play a role in oxygen sensing [47, 48, 57]. However, the involvement of individual NOX isoforms and whether inhibition or rather enhancing NADPH oxidase activity during this phase is the appropriate therapeutic approach is unclear.

During reperfusion, NADPH oxidase inhibition could be a new therapeutic option in acute events, such as stroke or MI, if it is possible to intervene exactly during this period. Patients diagnosed with ischemic stroke or MI could be treated with NADPH oxidase inhibitors even before measures to establish reperfusion are taken. Also, in patients in whom reperfusion has already occurred endogenously, treatment with an NADPH oxidase inhibitor could be initiated in the early phase of reperfusion. For example, in brain IRI, inhibition of NOX4 post-reperfusion is beneficial in mice after an ischemic stroke [62]. NOX4 has been found in mitochondria [40]. As mitochondrial ROS likely have detrimental roles in the acute damaging phase after I/R of the heart [124], reducing mitochondrial ROS generation by NOX4 inhibition is an attractive strategy. NOX2 may also be a good target in this condition, since an inflammatory reaction and disturbed BBB promotes the influx of neutrophils into the ischemic brain area [85]. However, data regarding NOX2 are conflicting [88]. In heart IRI, even more NOX isoforms may play a role [22]. Importantly, NOX5, the only NOX isoform, which is activated by calcium, is a potential target to reduce oxidative stress in VSMC [39, 140] as well as in EC [38] and possibly also directly in the heart. Unfortunately, due to the lack of NOX5 in rats and mice, no in vivo data on the role of NOX5 in animal models of MI are available.

During the third post-reperfusion phase, ROS appear to be needed for angiogenesis [15]. However, at the same time, they can be detrimental in promoting hypertrophy or dilatation. Here, not only the amount of ROS and the place where they are produced, but also the underlying causes of remodeling seem crucial, as is shown by divergent results in different pressure overload models [141, 142]. In chronic ischemic diseases, such as peripheral artery disease (PAD), the inhibition of NADPH oxidases could lead to an even more impaired blood flow [114] via interfering with angiogenesis. Here, NADPH oxidase-stimulated angiogenesis could lead to a better preserved vascularization and oxygen delivery within the peripheral tissues [67]. Thus, the activation of specific NOX isoforms at specific locations may here be a therapeutic option for PAD patients. Since NOX1, NOX2, and NOX4 are expressed in both VSMCs and ECs [22], they all seem to be possible targets in chronic ischemic disease states.

NADPH oxidase inhibitors

As is clear from the discussion in the previous chapter, complete inhibition of NADPH oxidase activities may not be a good strategy. Rather, it is important to delineate the precise roles of individual NOX isoforms, and it may turn out that one isoform should be inhibited, while another should be left untouched or even activated. Isoform-specific NOX inhibitors developed as therapeutic drugs could be used at specific time points during IRI.

Due to the high degree of structural similarity among the NOX enzymes [22], the development of isoform-selective NADPH oxidase inhibitors seems difficult. However, to avoid unforeseeable and potentially severe side effects of unselective NOX inhibition, this is highly desired. Currently, there is only one NOX isoform-specific inhibitor published, which is the NOX2 inhibitory peptide gp91ds-tat [143]. However, peptides are not orally bioavailable and probably do not show suitable pharmacokinetic parameters for clinical applications. Thus, small-molecule inhibitors are better suited. Nevertheless, published small-molecule NADPH oxidase inhibitors with some degree of selectivity for one NOX isoform, such as ML171 [144] and M090 [145] (NOX1 selectivity), do not seem to have satisfying pharmacokinetic properties. Other inhibitors, both isoform-selective and isoform-unselective, have not yet been thoroughly investigated for off-target effects and toxicity, for example, fulvene-5, VAS2870, M090, and to some degree, ML171 [82, 144–146]. VAS2870 has been shown to reduce ROS production in vitro [147] and to diminish brain damage after stroke in vivo [62]. However, initially, VAS2870 seemed to be specific for NADPH oxidases [138], but recently, potential off-target effects of VAS2870 have been reported [148] with thiol alkylation as the likely mechanism of action of VAS2870-mediated NADPH oxidase inhibition. Clearly, the importance of this off-target alkylation or possible other off-target effects of VAS2870 need further analysis. The first inhibitor showing suitable toxicity and high oral bioavailability was the NOX1 and NOX4 inhibitor

GKT136901 [149], which was further optimized to GKT137831. The latter compound is now in a phase I clinical study for the indication diabetic nephropathy.

In summary, (isoform)-specific NOX inhibitors or stimulators with a good pharmacodynamic and pharmacokinetic profile would be a significant step forward in the treatment of diseases, in which IRI plays a role.

Summary and outlook

Isoform-selective inhibition of NADPH oxidases seems to be a promising strategy to treat stroke and MI. Instead of scavenging ROS with antioxidants, selective blockade of ROS production at pathologically important sites may open new doors for therapies. Timing of selective NOX blockade may be of utmost importance because of the opposing effects in the different phases of IRI. In the post-reperfusion phase, stimulation of NADPH oxidases might even be an option. The same may hold true for more chronic ischemic diseases, in which ROS likely mediate angiogenesis. Thus, it is clear that unraveling the precise sequence of events and differential roles of the NADPH oxidase–ROS axis is crucial for successful therapeutic clinical translation. Keeping in mind the double-edged sword that NADPH oxidases present, we must defeat the detrimental effects and possibly embrace the beneficial effects at the same time.

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